Cancer Chemo–Preventive Potential of Berberine Conjugated Silver Nanoparticles

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Tumor-associated macrophages (TAMs) play a critical role in tumor progression, influencing growth, metastasis, and resistance to therapies like chemotherapy and radiotherapy(Tan et al., 2021). These macrophages adapt within the tumor microenvironment (TME) to form either pro-tumorigenic M2-like or anti-tumorigenic M1-like phenotypes, with M2-like TAMs commonly associated with immunosuppression and tumor promotion(Yang et al., 2023). Understanding the dual nature of TAMs is essential for developing targeted therapeutic strategies.

TAMs in Tumor Growth and Chemoresistance

The influence of TAMs on chemotherapy varies depending on the cancer type and therapeutic agent. TAMs have been linked to mechanisms of drug resistance, such as the secretion of CCL5, which activates STAT3 signaling and induces Nanog overexpression, as seen in chemoresistance studies (Malfitano et al., 2020). In prostate cancer, TAMs contribute to resistance by secreting CXCL12, which enhances cancer cell survival and decreases chemotherapy sensitivity. TAMs also interact with tyrosine kinase inhibitors, such as EGFR-TKIs, which are employed in non-small cell lung cancer (NSCLC), indicating the need to address TAM-mediated resistance in novel cancer therapies (S. Chen et al., 2023; Duan & Luo, 2021).

TAM-Targeted Therapeutic Strategies

Addressing the impact of TAMs in the TME involves various approaches targeting their recruitment, phenotype, and function. Therapeutic strategies are broadly classified into five categories:

1. **Enhancing TAM Phagocytic Activity**

The CD47-SIRPα axis serves as a pivotal target for enhancing macrophage-mediated phagocytosis. CD47 inhibitors like Hu5F9-G4 and SIRPα-Fc fusion proteins (e.g., TTI-621)

have shown efficacy in improving TAMs' ability to recognize and eliminate tumor cells. Innovative therapies, such as chimeric antigen receptor macrophages (CAR-Ms), further amplify TAM-mediated phagocytosis and cytokine release (Klichinsky et al., 2020).

2. **Depleting M2-Like TAMs**

Selective depletion of M2-like TAMs through bisphosphonates, such as zoledronate, has demonstrated efficacy in reducing tumor growth. Additionally, novel compounds like TD-92 enhance immune checkpoint blockade by targeting TAMs while preserving M1-like macrophages, offering potential synergistic anti-tumor effects (Shih et al., 2021).

3. **Preventing TAM Recruitment**

The recruitment of TAMs into the TME is mediated by signaling axes like CSF1/CSF1R and CCL2/CCR2. Inhibitors such as PLX3397 and PF-04136309 have shown promising results in clinical trials by reducing TAM infiltration and improving T-cell activation, thereby enhancing anti-tumor immunity(Benner et al., 2020; Noel et al., 2020).

4. **Reprogramming TAMs**

Reprogramming M2-like TAMs into M1-like phenotypes restores their anti-tumor activity. Strategies involve targeting CSF1R, TLRs, and CD40, alongside employing drugs like RRX-001 and miRNAs. These interventions enhance the efficacy of immune checkpoint inhibitors and address TME immunosuppression (Xiang et al., 2021).

5. **Nanotechnology-Based Approaches**

Nanoparticles (NPs) offer a sophisticated method for TAM targeting by enhancing drug delivery precision and minimizing systemic toxicity. For instance, STAT6 inhibitors delivered via NPs effectively reprogram M2-like TAMs into the M1 subtype. This approach not only

inhibits tumor growth but also mitigates immunosuppressive effects within the acidic TME (Xiao et al., 2020).

Implications for Cancer Therapy

The ability to manipulate TAMs opens a frontier in precision oncology, with approaches ranging from depletion to reprogramming and drug delivery via nanotechnology. Combining TAM-targeted therapies with conventional treatments such as chemotherapy, radiotherapy, and immunotherapy holds promise for improving patient outcomes. By targeting TAMs' multifaceted roles in the TME, researchers aim to mitigate their contribution to therapy resistance and immunosuppression while enhancing anti-tumor immunity.

The Rationale of the Study

Although berberine and silver nanoparticles hold significant promise for targeted cancer therapy, research in this field remains limited, leaving substantial opportunities for further exploration. The addition of PVP as a stabilizing and capping agent further enhances the functionality of these nanoparticles by improving their stability, size uniformity, and dispersion, while preventing aggregation. The combination of berberine, a natural alkaloid with known anticancer properties, and silver nanoparticles, valued for their antimicrobial and cytotoxic effects, represents an innovative approach to cancer treatment. When functionalized with folate for targeted delivery, these nanoparticles could offer enhanced specificity to cancer cells overexpressing folate receptors, reducing side effects on healthy tissues. However, the lack of comprehensive studies has resulted in significant gaps in understanding their interaction with biological systems. Investigating these aspects could unveil the full potential of P–AgNPs–BBR–Fo in overcoming challenges such as nanoparticle stability, targeted delivery efficiency, and controlled release of therapeutic agents. Rigorous research into their toxicity profiles, bioavailability, and scalability for clinical use is also essential. Addressing these gaps

could pave the way for the development of effective and safe nanotherapeutics a valuable tool in cancer treatment.

Objectives of the Study

a. Loading of berberine to silver nanoparticle and characterization of berberine silver nanoparticle
b. Study of inhibitory effects of Berberine silver nanoparticle on cancer cells.

The following studies were performed to achieve these objectives:

Poly(vinylpyrrolidone)-capped silver nanoparticles (P-AgNPs) were synthesized by standardizing with PVP, glucose, and KOH. These P-AgNPs were then loaded with berberine and conjugated with folate. The resulting folate-tagged berberine nanoparticles were characterized using UV-Vis spectroscopy, SEM, FTIR, XRD, and DLS with zeta potential measurements. These folate-tagged berberine nanoparticles were subsequently evaluated for various biological activities, including antioxidant, antibacterial, and anticancer activities.

INTRODUCTION

Cancer originates from changes in one or a few cells among the body's trillion cells(Rycaj & Tang, 2015). This alteration disrupts the signals that regulate cell division, potentially leading to uncontrolled growth and the formation of tumors. While solid tumors typically form as localized growths, some cancers, such as leukemia, arise from blood cells, resulting in an accumulation of malignant cells in the bloodstream and bone marrow(Hanahan & Weinberg, 2000). The initial stages of cancer are driven by genetic changes that disrupt normal cellular processes.

Genetics is central to cancer development, as DNA undergoes constant damage, repair, and resynthesis(Loeb & Loeb, 2000). In healthy cells, these processes are tightly regulated, ensuring stability. However, cancer cells often exhibit genetic instability due to mutations in genes responsible for maintaining genomic integrity. This instability can manifest as chromosomal aberrations, gene amplification, or aneuploidy, which contribute to the diverse genetic profiles of tumors(Ferguson et al., 2015). DNA damage, arising from both external and internal sources, plays a critical role in the mutation accumulation that drives carcinogenesis. Mutations in critical pathways, loss of cell cycle checkpoints, and defects in apoptosis exacerbate this instability, highlighting the intricate genetic changes underpinning cancer(Ashouri et al., 2023).

Reactive Oxygen Species (ROS) significantly influence cancer biology, functioning as both a driver of tumor progression and a potential therapeutic target. ROS are produced endogenously, such as through mitochondrial activity, or externally, such as from inflammation. Elevated ROS levels in cancer cells induce DNA damage, promoting mutations and disrupting cellular homeostasis(de Almeida et al., 2022). Cancer cells develop robust antioxidant defences to counterbalance their high ROS levels. Exploiting this distinction offers opportunities for

therapeutic interventions aimed at targeting ROS-dependent signaling pathways. (Mukherjee et al., 2024)

ROS impact cancer through pathways such as MAPK/ERK and PI3K/Akt, which regulate growth, survival, and inflammation. For instance, ROS activate Erk1/2, promoting proliferation and survival in breast and melanoma cancers(Arfin et al., 2021). Similarly, the PI3K/Akt pathway, modulated by ROS, inhibits apoptosis, enhancing cancer cell resilience. The IKK/NF-κB pathway, driven by ROS, plays a critical role in inflammation and cancer progression, underscoring the multifaceted role of ROS in tumor biology(He et al., 2021).

The tumor microenvironment (TME) also plays a pivotal role in cancer progression. It comprises immune cells, fibroblasts, endothelial cells, and extracellular matrix components (Goenka et al., 2023). While immune cells like T cells and dendritic cells typically suppress tumor growth, others, such as tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells, promote tumor development by dampening antitumor immunity(Balta et al., 2021). TAMs, often polarized toward an M2 phenotype, facilitate tumor progression through angiogenesis, immune suppression, and resistance to therapies. Pathways like PI3K/Akt/mTOR are crucial in TAM polarization, presenting potential targets for therapeutic intervention(Xia et al., 2023).

Inflammation further complicates cancer progression. Chronic inflammatory conditions, such as pancreatitis and colitis, increase cancer risk by altering the TME. For example, in pancreatic cancer, inflammation promotes acinar-to-ductal metaplasia (ADM), a precursor to cancerous lesions(Marstrand-Daucé et al., 2023). Macrophages play a vital role by secreting inflammatory cytokines that drive cellular reprogramming and tumor initiation. These insights suggest targeting inflammation and macrophage activity as potential strategies to curb tumor progression.

Nanotechnology

"Nanotechnology is an advanced area of research that allows for the production of a wide class of materials, including particulate materials with at least one dimension of less than 100 nanometers (nm)"(Saleh, 2020). As per the United States National Nanotechnology Initiative, nanotechnology involves comprehending and directing matter within the range of approximately 1 to 100 nm, leading to distinctive properties linked to size. Nevertheless, there are slight variations in this explanation based on different governing bodies like the "European Commission" or the "International Organization for Standardization (ISO)" Technical Committee 229. In certain cases, materials within the range of 1000 nm are also classified as nanomaterials. Given the dynamic nature of this field, the terminology is regularly revised, most recently through the establishment of an international working group that is creating a consistent labeling system for nanoscale materials(Leon et al., 2020).

Berberine Silver Nanoparticles: A Synergistic Approach

The convergence of Berberine's (BBR) therapeutic properties with the attributes of AgNPs holds the potential for an innovative approach to cancer prevention. BBR's anticancer properties, when integrated with the unique capabilities of NPs, could pave the way for targeted and efficient chemo-preventive interventions.

MATERIALS AND METHODS

Chemicals

Silver nitrate($AgNO_3$), poly(vinylpyrrolidone (PVP), Glucose, Potassium Hydroxide (KOH), Berberine(BBR), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2-Azino-bis (3ethylbenzothiazoline-6-sulphonic acid)(ABTS), Ascorbic acid, Nutrient agar, Quercetin, Streptomycin, Dimethyl Sulfoxide (DMSO), Amikacin, Flucanazole , Potato dextrose agar

Silver nanoparticles were synthesized through a green chemistry approach following the method described by Chen et al. (Q. Chen et al., 2017). Silver nitrate ($AgNO_3$) served as the silver source, glucose acted as the reducing agent, poly(vinylpyrrolidone) (PVP) was used as the capping agent, and potassium hydroxide (KOH) was added to maintain an alkaline pH. The synthesis involved preparing Solution A by mixing 15 mM AgNO3 with PVP in a 1.5:1 molar ratio in 50 mL of distilled water. Glucose (10 mM) dissolved in 25 mL of water was then introduced to Solution A at 60°C along with 10 mM KOH, and the mixture was stirred for 10 minutes. The resultant P-AgNPs were collected by centrifugation at 11,500 rpm for 20 minutes, washed with distilled water, and dried. For the loading of berberine (BBR), 10 mg of BBR was mixed with 10 mg of P-AgNPs in 5 mL of dimethyl sulfoxide (DMSO), stirred overnight, and then subjected to dialysis in distilled water for 24 hours. The final P-AgNPs-BBR (PBR) product was centrifuged at 11,500 rpm to remove unbound substances. The synthesis of FA-PEG@BBR-AgNP nanomaterial involved a multi-step process starting with the preparation of Folate-Polyethylene Glycol (FA-PEG). Folate was activated using DCC/NHS in dimethyl sulfoxide (DMSO) and then reacted with PEG-bisamine to form FA-PEG. The FA-PEG was then used to functionalize P-AgNPs with Berberine (BBR) to produce FA-PEG@BBR-AgNPs. This was achieved by mixing FA-PEG amine with P-AgNPs-BBR in a phosphate-buffered saline solution, followed by centrifugation and washing to remove excess FA-PEG and unreacted components. Characterization of the P-AgNPs, P-AgNPs-BBR, and FA-PEG@BBR-AgNPs nanoparticles was performed using UV-visible absorbance Spectroscopy with a Shimadzu UV-1800 instrument, Scanning Electron Microscopy (SEM) with ZEISS EVO 15, and Energy Dispersive X-ray Spectroscopy (EDX) with Oxford-AZtec. The X-ray Diffraction (XRD) patterns were obtained using an Empyrean 3rd generation Malvern PANalytical instrument, and Dynamic Light Scattering (DLS) and Zeta Potential measurements were conducted using the Anton-Paar 3 LiteSizer™ 500 and Malvern ZetaSizer,

respectively. Fourier Transform Infrared Spectroscopy (FTIR) was employed using a Nicolet 6700 spectrometer to analyze molecular vibrations and interactions.

Antioxidant activity of the P-AgNPs, and P-AgNPs-BBR, were assessed using the DPPH radical scavenging assay and the ABTS assay as described by Hulikere et al. For the DPPH assay, various concentrations of P-AgNPs, and P-AgNPs-BBR (20, 40, 60, 80, 100 µg/mL) were mixed with 2 mM DPPH and incubated for 15 minutes, with absorbance measured at 517 nm. In the ABTS assay, sample concentrations (20, 40, 60, 80, 100 µg/mL) were combined with ABTS and potassium permanganate, and absorbance was recorded at 740 nm. Antimicrobial efficacy was tested against bacterial strains (Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa) and the fungus Aspergillus sp using the well-diffusion method, with inhibition zones measured to evaluate effectiveness. For cellular assays, Raw 264.7 macrophage cells were cultured in DMEM supplemented with fetal bovine serum and antibiotics at 37°C in a 5% CO2 atmosphere. Cell proliferation was evaluated using the MTT assay, where cells treated with FA-PEG@BBR-AgNPs or LPS were tested for viability after 24 hours. Cell morphology was examined under a microscope, and ROS generation was quantified using the DCF-DA probe. Nitric oxide levels were measured using the Griess reagent, and phagocytic activity was assessed with neutral red uptake. Gene expression was analyzed via quantitative Real-Time PCR (qRT-PCR) with GAPDH as the internal control, and Western blot analysis was performed to investigate protein expression changes related to inflammatory responses.

RESULTS AND DISCUSSION

In our current investigation, we developed a rapid and eco-friendly one-pot method for synthesizing AgNPs. AgNPs were chosen for their significant biological properties, including their potential to protect the gastrointestinal tract. In this synthesis, dextrose served as the

reducing agent while PVP acted as the capping agent. The reduction of Ag+ ions to their elemental state occurred efficiently at the boiling point of water (80°C). PVP-capped AgNPs are noted for their lower toxicity and better biocompatibility, making them suitable for medical applications. The resulting nanoparticles displayed promising spasmolytic, antibacterial, and immunohistochemical activities without exhibiting cytotoxic effects. Berberine (BBR), a positively charged cation, was employed to bind to the negatively charged PVP-coated AgNPs through electrostatic interactions. To facilitate the connection of folic acid (FA) with BBR-AgNPs, PEG-amine was used as a linker through hydrogen bonding, elucidating the reaction mechanism for the creation of FA-PEG@BBR-AgNPs.

The synthesis of P-AgNPs was achieved through the reduction of silver ions in an alkaline aqueous solution containing polyvinylpyrrolidone (PVP) and dextrose. This process was visually confirmed by the color change from colorless to yellow, indicative of the formation of P-AgNPs. The presence of nanoparticles was further substantiated by a UV-Vis absorption spectrum showing a peak at 451 nm, corresponding to the surface plasmon resonance of the small-sized P-AgNPs. Analysis of different concentrations of $AgNO_3$ (3, 5, 10, 15, and 20 mM) revealed that the absorbance increased up to 15 mM, but beyond this concentration, the absorbance decreased, likely due to enhanced agglomeration and coalescence of nanoparticles due to increased ionic strength. Additionally, varying the PVP to AgNO3 ratio affected the size and distribution of nanoparticles, with a 1:1.5 ratio achieving optimal dispersion and narrow size distribution, as shown by SEM images. FTIR indicated that PVP effectively capped the nanoparticles, while EDX confirmed a high silver content in the 1:1.5 P-AgNPs. However, these P-AgNPs demonstrated limited antioxidant and antibacterial activities compared to other formulations, with the highest PVP ratio showing the most effective stabilization but minimal biological efficacy.

Polyvinylpyrrolidone-coated berberine-loaded silver nanoparticles (PBR) were synthesized and characterized to evaluate their potential as therapeutic agents. The UV-Vis spectrum of PBR revealed a peak at 451 nm, consistent with the presence of silver nanoparticles, while the characteristic peaks of BBR shifted upon interaction with AgNPs, indicating successful loading of BBR onto the nanoparticles. SEM imaging showed that PBR particles had a spherical, non-aggregated morphology with a mean diameter of approximately 78 nm, while EDX analysis confirmed the presence of Ag, C, O, Na, and Cl, with Ag being the predominant element. XRD analysis identified the characteristic face-centered cubic structure of metallic silver in PBR. DLS measurements revealed an average hydrodynamic size of 284 nm, which is larger than the size observed in SEM due to the presence of the stabilizing PVP layer. Zeta potential analysis showed a negative value of -23.7 mV, indicating good stability of the nanoparticles. The FTIR spectrum of PBR revealed key functional groups associated with both PVP and BBR, with significant peaks corresponding to C=O and C-H bonds, indicating successful capping and BBR attachment. In vitro drug release studies demonstrated that BBR was released in a pH-dependent manner, with a higher release rate at more acidic pH. Despite showing some antioxidant and moderate antifungal activities, PBR's effectiveness was still less than that of standard agents, highlighting the need for further optimization to enhance its therapeutic potential.

The synthesis and characterization of FA-PEG@BBR-AgNPs were evaluated their potential as therapeutic agents. The synthesized materials were performed using various analytical techniques. The UV-Vis spectra, demonstrated characteristic absorption peaks at 229, 260, 344, and 426 nm for BBR, with similar peaks observed in FA-PEG@BBR-AgNPs, confirming successful BBR attachment. XRD analysis revealed a face-centered cubic structure of AgNPs, with peaks corresponding to Miller Indices (111), (200), (220), and (311). SEM images indicated that the AgNPs had a spherical shape with sizes ranging from 60 to 90 nm and

exhibited some aggregation. The size distribution and zeta potential measurements confirmed that the AgNPs had a peak size of 420 nm and a zeta potential of −30.1 mV, indicative of stable dispersion in solution. Biological assays demonstrated that while BBR, AgNPs, and their conjugates improved cell viability at concentrations up to 100 μg/mL, AgNPs alone did not significantly affect cell viability. The treatment of LPS-induced RAW 264.7 macrophages showed that FA-PEG@BBR-AgNPs and AgNP-BBR significantly reduced ROS production, NO release, and pro-inflammatory cytokine levels, while also enhancing phagocytic activity. Gene expression analyses revealed that FA-PEG@BBR-AgNPs effectively down-regulated iNOS, GPX1, COX-2, and other inflammatory markers. Overall, this study highlights the potential of PVP-capped AgNPs and their conjugates for therapeutic applications, including their role in reducing oxidative stress and inflammation in macrophage cells.

Summary and Conclusion

PVP-stabilized silver nanoparticles (P-AgNPs) were successfully synthesized by the reduction of silver ions using glucose in an alkaline medium. The study demonstrated that varying the concentration of $AgNO_3$ and PVP significantly impacted the size, shape, and dispersion of the nanoparticles. The UV-Vis, SEM, XRD, FTIR, and DLS analyses confirmed the formation of crystalline, monodisperse nanoparticles with sizes ranging from 40 to 100 nm, depending on the $AgNO_3$ to PVP ratio. The optimal synthesis condition was achieved with a 1:1.5 ratio of $AgNO_3$ to PVP, resulting in the smallest and most uniform nanoparticles. The synthesized P–AgNPs–BBR demonstrated successful formation and characterization through various techniques, including UV-Vis, SEM, EDX, XRD, DLS, FTIR, and NMR, confirming the effective reduction of silver ions and the successful loading of BBR onto AgNPs. The nanoparticles exhibited desirable size, stability, and controlled release properties, with a pH-dependent release pattern, highlighting their potential for targeted drug delivery. While the P–

AgNPs–BBR formulation showed significant antioxidant activity and moderate antifungal properties, its antibacterial effects were less pronounced compared to the standard antibiotic amikacin. AgNPs functionalized with BBR and FA-PEG (AgNPs-BBR-Fo) were characterized through UV-vis spectra, XRD, SEM, FT-IR, and NMR confirming successful functionalization and stable dispersion of nanoparticles. Biological assays revealed that AgNPs-BBR-Fo significantly enhanced cell proliferation and morphology in M2 macrophages, alongside an increase in reactive oxygen species (ROS) production, indicating a potential mechanism for tumor treatment. Additionally, AgNPs-BBR-Fo suppressed nitric oxide (NO) production and modulated the expression of inflammatory cytokines like TNF-α, IL-1β, and IL-6, while promoting immune responses by upregulating MIG and downregulating RORγt. The AgNPs-BBR-Fo also affected key genes involved in inflammation, apoptosis, and antioxidant defence pathways, demonstrating its potential as a modulator of macrophage polarization and a therapeutic agent in cancer immunotherapy. These findings suggest that AgNPs-BBR-Fo holds promise for future applications in targeting inflammation and modulating immune responses in the tumor microenvironment.

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